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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
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Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

PULSE-MEDIUM PERFUSION BIOREACTOR WITH IMPROVED MASS TRANSPORT FOR MULTIPLE 3D CELL CONSTR

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Respectfully submitted,

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Number 2 of 2

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Pulse-Medium Perfusion Bioreactor with Improved Mass Transport for Multiple 3-D Cell Constructs

Background

Tissues in the body overcome issues of oxygen and nutrient distribution by containing spaced capillaries that provide conduits for convective transport of nutrients and waste product to and from the tissues. The three-dimensional cell constructs that are developed *ex-vivo*, usually lacks the vascular network that exist in normal vascularized tissues. Thus, the gas and nutrient supply to the scaffold-seeded cells depends merely on mass diffusion. To improve mass transport, we employ bioreactors, which portray different patterns of fluid dynamics and vessel geometry. Ideally these bioreactors must allow for control over the physicochemical environment (e.g., pO_2, pH, pCO_2, shear stress), allow aseptic feeding and sampling to follow issue development, and maximize use of automated processing steps to increase reproducibility. Standard bioreactor technologies are well suited to address many of the issues for cell expansion, but they have limitations when used for the other tissue engineering applications. In particular, the cultivation of three-dimensional tissue constructs place great demands on the mass transport function (e.g., nutrient distribution). In addition, it may be necessary to simultaneously culture multiple cells types for a certain application, and this may require more complex bioreactor designs.

Previously, we reported the cultivation of cardiomyocytes constructs in the rotating cell culture systems (RCCS), which were developed by NASA. In RCCS, the operating principles are: (1) solid body rotation about a horizontal axis which is characterized by extremely low fluid shear stress and 2) oxygenation by active or passive diffusion to the exclusion of all but dissolved gasses from the reactor chamber, yielding a vessel devoid of gas bubbles and gas/fluid interfaces. We showed that pO_2, pH and pCO_2 levels were maintained in the vessels to a better extent and allowed an aerobic respiration for a large number of cells compared to the static vessel. Cultivation of cardiac cell constructs in RCCS produced engineered cardiac tissues with improved cellularity, cell metabolism and expression of muscle specific markers. Although the above bioreactors provided a near homogenous external environment for the 3-D cell constructs, the extent of medium perfusion into the core of the engineered tissue is still limited due to the absence of capillary network in the developing tissue. As a result, the cells at the center of the 3-D engineered tissues do not benefit from the external dynamic fluid.

To solve this issue, we developed a novel pulse-medium perfusion bioreactor (PMPB). The system pumps medium directly through the 3-D cell-seeded scaffolds to enhance mass transfer into the developing tissue. Furthermore, mechanical stimuli are provided by an interval flow of medium and provide pulses of medium in a similar fashion to the pumping activity of the heart.

Description of the Pulse-Medium Perfusion Bioreactor (PMPB)

The concept leading the bioreactor design is improving mass transport into the 3-D cell constructs via perfusion while mimicking the heart physiology, which sends pulses of oxygenated blood to the different tissue in the body. Perfusion and pulsation is based on dynamics of the flowing fluid and is achieved by forcing the culture

medium directly into the 3-D cell constructs via specially designed proprietary flow directing net. The rate of the fluid flow is controlled by a computerized peristaltic pump.

The essential components of the bioreactor system (Fig 1) include: 1) A bioreactor body. A chamber designed to enable the desired environment for the cultivated 3-D cell constructs. The geometry of the chamber plays a crucial role in controlling fluid dynamics and enhancement of medium perfusion through the cell construct (its internal components are described below- Figure 2). 2) An oxygenated medium reservoir built of plexiglass. The oxygenator enables the control over the dissolved gases in the culture medium. 3) A peristaltic pump. A computer connected to the pump achieves the control over perfusion and mechanical stimuli. 4) Heat exchanger, maintains a constant temperature (37°C).

The medium flows from the peristaltic pump into the bioreactor and proceeds to the reservoir, it is then oxygenated and pumped back to the bioreactor as shown in **Figure 1.**

A- Bioreactor body
B- Reservoir and oxygenator
C- Heat exchanger
D- Peristaltic pump
E- Gas container

I.

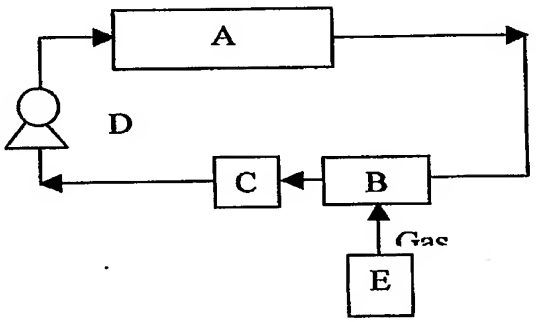

Figure 1II
Picture of the bioreact of system

Figure 1 The PMPB system. **I.** Medium circulation within the system. Arrows indicates the direction of flow. **II.** The whole system when operated.

Bioreact r body design

The bioreactor body consists of two identical halves (Figure 2A). The halves are connected to each other and function as the inlet and outlet to the cell compartment wherein the 3-D cell constructs are placed. The inlet and outlet angles of the identical

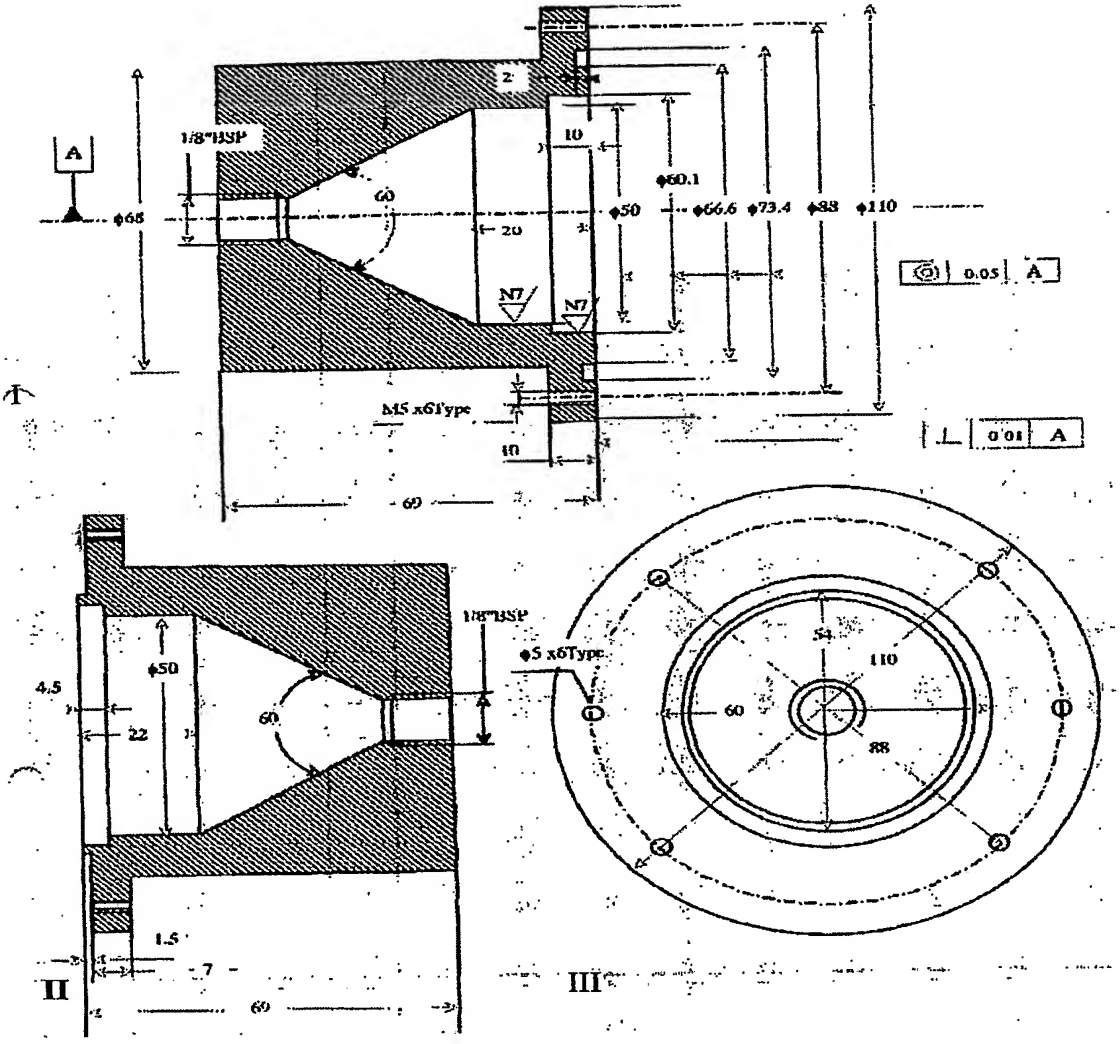

Figure 2 A: Shematic of the bioreactor body. **I** and **II** are a side-view pictures of the identical halves of the chamber, **III** is cross section in the interface between the two halves (dimensions in mm).

halves were designed to be with an angle of 30° to prevent the break down of the incoming flow, thus avoiding turbulancy. In addition, a distributing-fluid-mesh is located at each inlet/outlet halves.

The cell compartment can occupy **multiple** 3-D cell constructs; the number depends on the size of the individual constructs (for constructs with a diameter of 5mm, approaximately 35 cell constructs) (Figure 2B). The compartment is constituted of two nets; in-between them the cell constructs are fixed to avoid their dislocation. The selected net geometry ensures maximal exposure of the entire cell contructs to the perfused medium. This special design is different from the existing conventional nets, which mask the flow.

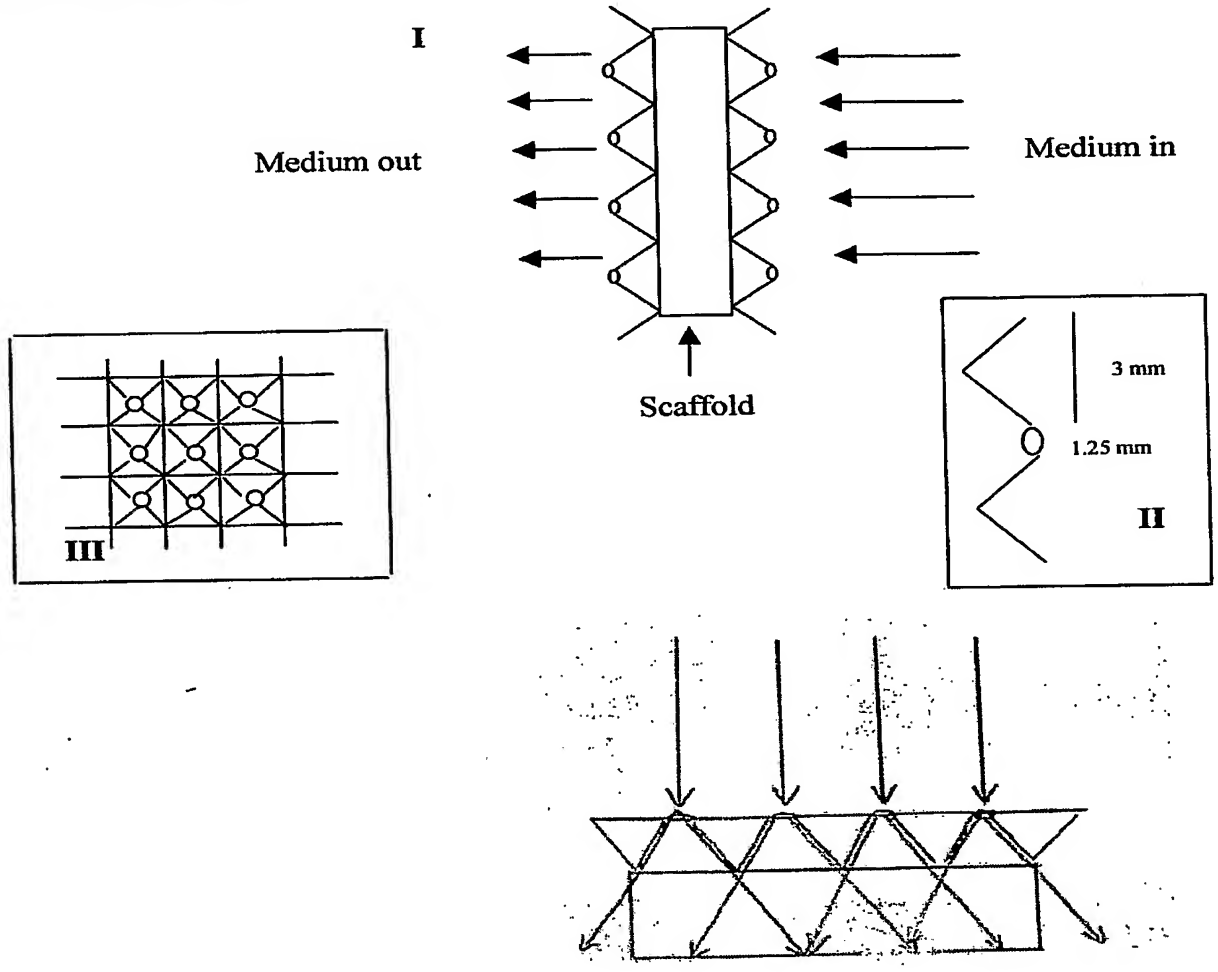

Figure 2 B Position of the nets inside the bioreactor's body. **I.** demonstrates the position of the cell constructs between the two fixing nets, in the cell compartment. Arrows indicates the direction of flow in the bioreactor body. **II.** describes the dimensions of the net. **III.** Top view of the net. **IV.** Shows that the cell constructs are fully exposed and perfused by the medium.

Classification of Fluid Flow in PMPB

In order to determine whether the flow in the bioreactor body is laminar or turbulent, Reynolds number was used:

$$Re = V * d / \nu$$

Where V is the medium's average velocity, d is the bioreactor's body diameter and ν is the kinematic viscosity. The average velocity can be calculated from the equation:

$$Q = V * A$$

Where Q is the flow rate, and A is the cross section area of the bioreactor body at the compartment area. Since Q is set to be 50 ml/min, and $A = \pi d^2/4$ where d is 0.05 m the cross section area is:

$$A = 1.96 * 10^{-3}\ m^2$$

Thus, the velocity in the scaffolds compartment is:

$$V = Q/A = 4.26 * 10^{-4}\ m/s$$

Since the kinematic viscosity of the culture medium at 37°C is very close to the water's kinematic viscosity at 37°C

$$\nu = 0.7 * 10^{-6}\ m^2/s$$

Thus, Reynolds number is:

$$Re = 30.46$$

In view of the fact that the calculated Reynolds number is less than 2000, the medium flow inside the bioreactor is laminar.

Profile of the flow velocity

The Navier-Stocks profile of a developed laminar flow velocity in a pipe (Figure 3) is according to the following equation:

$$Vz = \frac{(\Delta P)}{4L\mu} R^2 \left[1 - \left(\frac{r}{R}\right)^2\right]$$

Where V_z is the velocity at radius r, ΔP is the pressure gradient (through L), L is the length of the pipe, μ is the viscosity of the fluid, R is the radius of the pipe and r ranges between 0 and R.

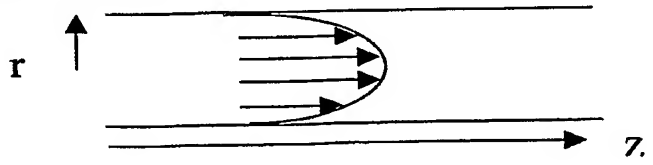

Figure 3 The velocity profile of a laminar developed flow in a pipe. Velocity changes along the radius r.

In order to avoid different stresses upon the cell constructs at different locations in the compartment (as a result of various velocities) an additional mesh was added. The mesh equally distributes the velocity in the pipe by breaking the developed flow and initiating it in a close distance from the cell constructs, thus providing an undeveloped laminar profile and an equal stress on the scaffolds (Figure 4).

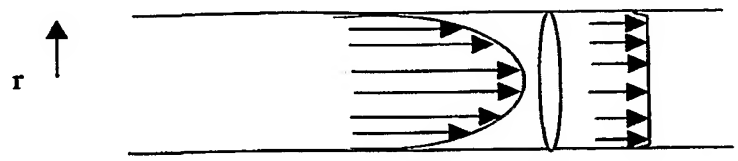

Figure 4 The velocity profile of a laminar flow in a pipe before and after a mesh is added.

Calculation of stress on the cell constructs

The stress induced upon the cell constructs in the bioreactor body, can be expressed by the following equation:

$$\tau = F_D / S$$

Where τ is the stress on each scaffold, F_D is the drag force acting on the scaffold and S is the surface area subjected to the stress.

F_D is calculated as followed:

$$F_D = 0.5\rho V^2 A C_D$$

Where ρ is the fluid density, V is the fluid velocity, A is the cross section area of the scaffold, and C_D is the drag coefficient, and in our case is 1.Thus:

$$F_D = 1.37 * 10^{-6}\ kg*m/s^2$$

Since S is $\pi r^2 + h* 2\pi r$ where h is the scaffold's height and r is its radius, the stress acting on the scaffold is:

$$\tau = 0.265\ dynes/cm^2$$

In our system, a change in the flow rate (Q) will lead to different stresses in an exponential rate on the scaffolds.

In PMPB, a computerized peristaltic pump provided mechanical stimulus via medium pulses. During operation, the peristaltic pump impelled 5 ml of the culture medium at a rate of 50 ml/min. The interval between each medium pulse was 0.2 sec. Such operation mimics heart beating.

The reservoir and xygenator design

The reservoir (750 ml) is made of plexiglass and a plexiglass tube penetrating to about 70% of its height. The tube serves as an oxygenator and it is set to preserve constant pH, PO_2 and PCO_2 in the culture medium. The tube is connected via a filter to a gas container with a composition of 21% O_2, 5% CO_2 and the balance N_2. The reservoir (Figure 5) consists of a gas outlet tube, to revoke the resisting pressure, medium inlet and outlet tubes (from and to the bioreactor body) and a medium sample collection outlet.

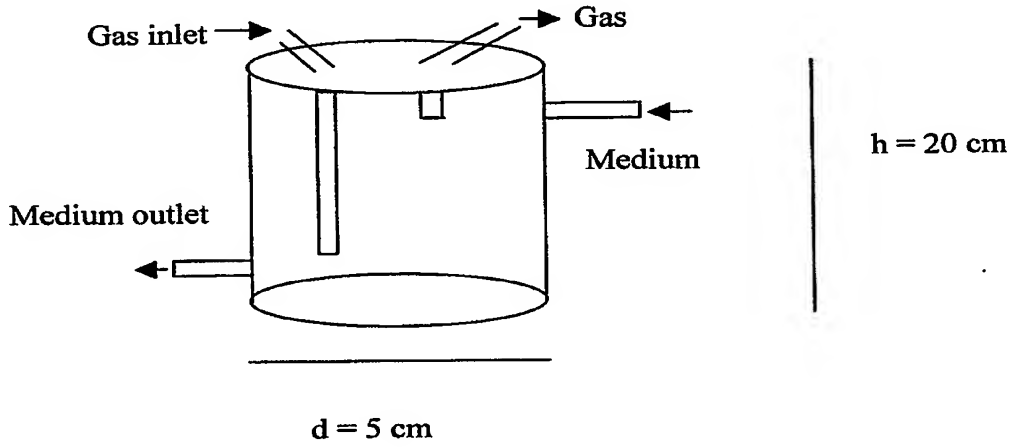

Figure 5 The oxygenated-reservoir. The arrows indicate the flow directions.

The oxygen mass transport from the incoming bubbles (through the tube) to the medium in the reservoir is defined as:

$$OTR = \frac{dC_o}{dt} = k_L a\left(C_o^* - C_o\right)$$

Where *OTR* is the oxygen transport rate, C_o^* is the oxygen concentration of the bubble medium interface, C_o is oxygen concentration in the medium bulk, k_L is the oxygen mass transport coefficient at the border layer of the bubble and the medium and a is the surface area of the bubble per its volume. Since a is difficult for detection, the parameter k_La is defined as the volumetric oxygen mass transport coefficient and can be calculated by various methods.

A control over the *OTR* can be achieved by a change in C_o^* or k_La. Since C_o^* is constant in the gas container, the variable parameter is k_La. In our system k_La can be changed by a change in the width of the gas tube, since it changes the size of the bubble, therefore induces a change in a. Another factor effecting the oxygen concentration in the medium is the gas hold-up and the sustention time of the bubble in the system. Gas hold-up is defined as:

$$G_H = \frac{V_g}{V_g + V_l}$$

Where G_H is the gas hold-up, V_g is the total volume of the bubbles within the reservoir and V_l is the volume of the liquid in the system. High G_H and sustention time can enhance oxygen mass transport to the medium, on one hand but on the other hand can increase CO_2 concentration within the bubble, thus decreasing the oxygen mass transport. Optimization of these parameters has lead to an efficient operation of the system.

Experimental proof for medium perfusion in PMPB

The ability of the medium to perfuse into the cell constructs in PMPB was investigated by following the distribution of 5-carboxyfluorecein (CF) in alginate cell constructs with the following dimensions, 5x2 mm, dxh. Perfusion was achieved using a peristaltic pump, which pumped the medium at a rate of 150 ml/min. Cell constructs placed under static conditions or in PMPB were supplemented with a medium containing 0.5% (w/v) CF. After 1 min, the cell constructs were harvested, frozen and longitudinally sliced to 0.5mm slices. The slices were viewed under a fluorescent microscope and photographed (Figure 6). There is clear evidence that mass transport in cell constructs subjected to a perfused medium is higher than in cell constructs cultivated in static medium.

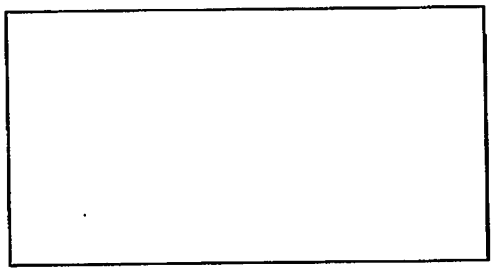

Figure 6 Fluorescence distribution in cell constructs cultivated under static (a) and perfusion (b) conditions

Cultivation of 3-D Cardiomyocytes in PMP Bioreactor

Effect of PMPB System on Cardiac Cell Viability

Alginate scaffolds (5*2 mm, d*h) seeded with cardiac cells ($7x10^5$ cells/scaffold) were cultivated within the PMPB system for 8 days. Samples (n=2-3 per data point) were taken every 2 days and MTT and Hoechst 33258 assays were performed to determine metabolic activity and DNA content. Figure 7 shows both metabolic activity and DNA content in relation to day 0, the results show high maintenance of over 90% of the initial cell number.

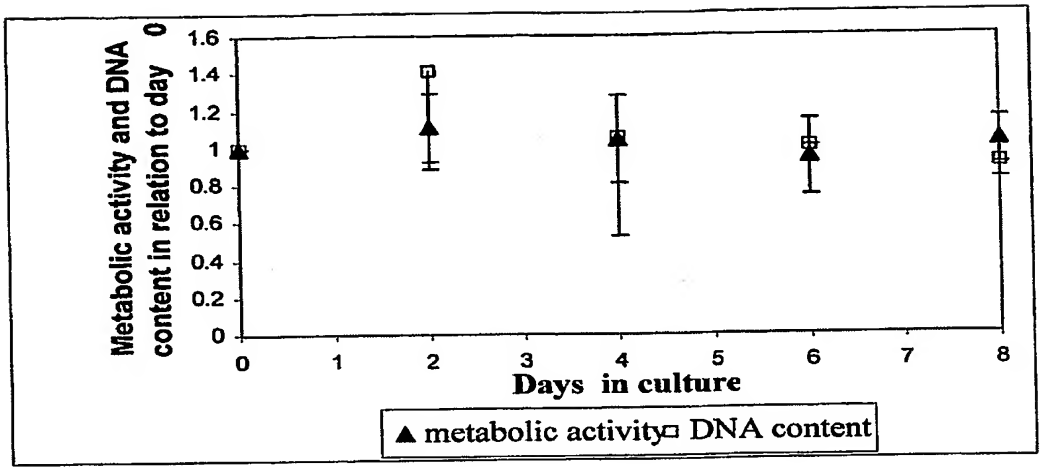

Figure 7 Cell viability and DNA content in the PMPB system. Metabolic activity and DNA content were evaluated using the MTT assay and Hoechst 33258 assay respectively

In the static cultivation (Figure 8), wherein one seeded scaffold was placed in a 1ml cm+ medium, the decline in cell number over time was pronounced, especially between days 4 & 8.

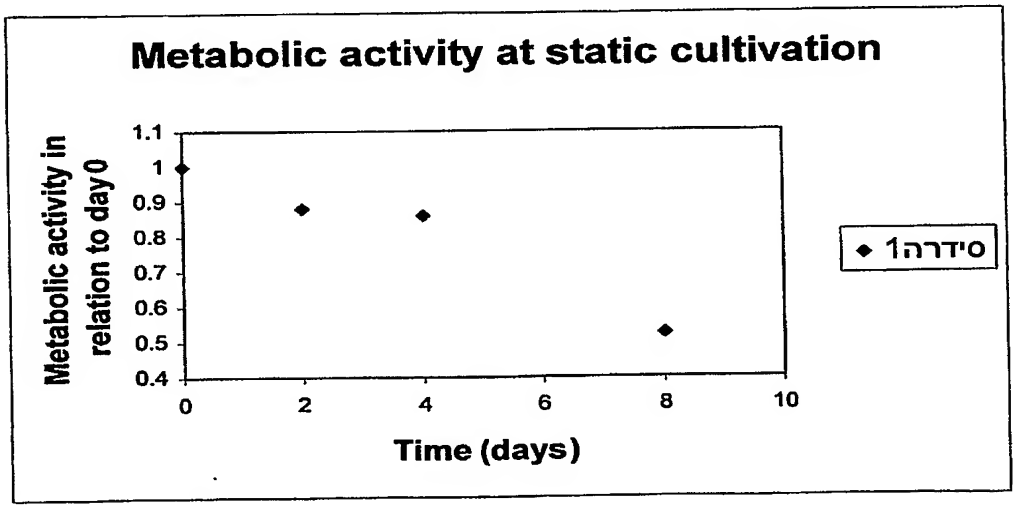

Figure 8 Cell viability throughout 8 days of static cultivation. Samples (n=2-3 per data point) were taken every 2 days and MTT assay was performed to each cardiac construct.

Metabolic Indices of Medium in PMPB System

A 0.5 ml CM+ medium from the PMPB system was sampled each day and analyzed for glucose and lactate using a gas blood analyzer. Figure 9 shows the yield of lactate on glucose ($Y_{L/G}$), calculated as a molar ratio of the produced lactate and utilized glucose. The ratio reveals aerobic cell metabolism.

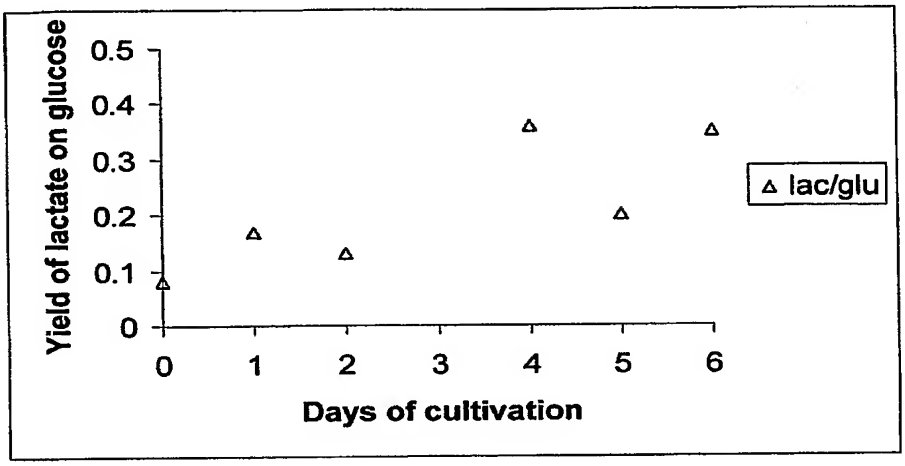

Figure 9 Yield of lactate on glucose ($Y_{L/G}$). Medium from the PMPB system was taken every day and analyzed by a blood gas analyzer. The values were calculated as molar ratios of produced lactate and utilized glucose

Effect of PMPB System on Cardiac Cell Distribution in Alginate Scaffolds

Staining the 7-day cardiac constructs cultivated in the PMPB system with both FDA (fluorescein diacetate) and PI (propidium iodide) (Figure 9) revealed viable cell clusters formed at the center of the cardiac construct.

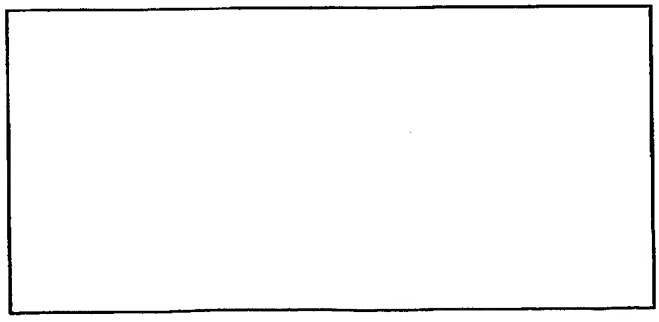

Figure 10 n show viable cell clusters at the center of the scaffold at day 7 of ı the PMPB system.

At day 14, the clusters appear to develop into a viable cardiac tissue with dimensions of 0.5 X 0.5 mm thickness (Figures 11 and 12)

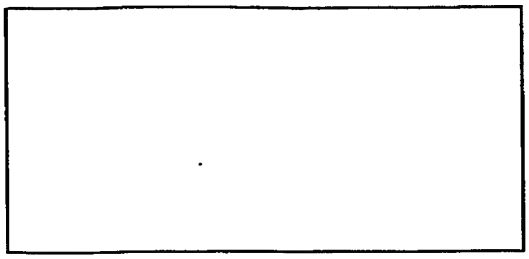

Figure 11 FDA-PI stain showing the developed of a large viable cardiac tissue at day 14 of cultivation in the PMPB system.

Hematoxylin-Eosin staining of thin sections (5 μm thickness) from the cardiac constructs cultivated in the PMPB system for 14 days revealed the formation of large cardiac tissue (Figure 12).

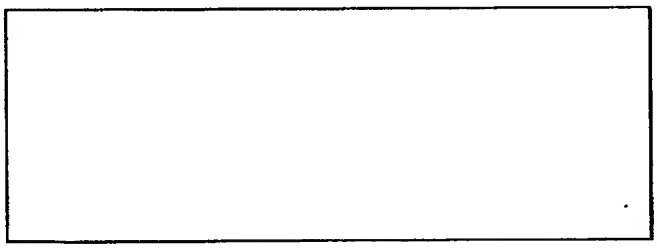

Figure 12 H&E stained thin cross-section of cardiac cell-seeded constructs, cultivated in the PMPB system for 14 days.

In summary, we described herein a novel bioreactor which was designed to enhance the direct perfusion of medium into 3-D cell constructs. In addition to the improved mass transfer, the ability to control medium flow enabled the application of medium in pulses, which imitates the phyiological situation in the heart. An oxygenated reservoir was incorporated into the system, to provide a better control over the dissolved gases in the culture medium and the internal elements within the bioreactor body maintained equivalent stress upon the scaffolds within. Finally, the PMPB system is capable of supporting multiple constructs and by that enabling scaling up the cultivation process. In the research phase, multiple assays can be performed at numerous time points, while using the PMPB system.

The collective results presented herein support the improved mass transport, which was translated into high cell viability (>90% of the seeded cardiac cells). With time, the cardiac cells organized into a functioning caridac muscle tissue. The yield of

lactate on glucose (YL/G), supported aerobic cell metabolism. Altogether, the results shown here are correlated with a better control over the oxygen and pH levels within the physiological range and the improved oxygen transport to the core of the cell-seeded scaffolds, leading to the formation of a long term viable cardiac constructs.

We envision that the PMPB system may be efficiently used for the engineering of thick and high cell density cardiac tissue constructs and that the ability to provide mechanical stimuli, via the medium pulses, may induce the formation of a functioning cardiac tissue.

Summary of the Invention

The invention comprises inter alia the following:

1. A bioreactor vessel, designed to enable a direct perfusion of the culture medium into 3D cell constructs. The vessel comprising two halves. When the halves are connected to each other, they create a compartment which encompass the 3-D constructs; the halves function as the inlet and outlet of the perfused liquid medium.
2. Each half is designed as a cylindrical vessel which becomes wider or narrower.
3. The vessel is built such that all angels 30^0 to prevent turbulent flow and eddies.
4. The first half consists of a flow distributor mesh to ensure laminar flow, such that the cell constructs placed, at the bioreactor cross section area, are subjected to equal velocity and stress. Similar flow distributor mesh is placed at the other half for reversible operation.
5. A fixed unique net is placed at the end of the front half of the bioreactor, and a similar removable net is placed at the second half. The latter can be replaced as a function of the scaffolds height, placed in-between these two nets.
6. The geometry of these two nets ensures fixation of the 3-D cell constructs in the cell compartment
7. The net is design so that it does not block the incoming liquid medium to reach each point in the entire cell constructs.
8. The bioreactor design allows medium perfusion into the entire cell seeded scaffolds.
9. The two halves of the bioreactor are pressed to each other by 6 screws and an O ring between the two halves for total sealing.
10. The direct flow of the medium into the cell constructs can be used to achieve different rate of medium delivery such as perfusion at pulsatile vs. continuous flow.
11. The bioreactor enables the cultivation of thick 3-D constructs (> 100 μm)

12. The medium perfusion allows cell disribution through the entire construct

13. The bioreactor enabled adequate mass and gas transfer into each cell in the construct.

Fig 1 II

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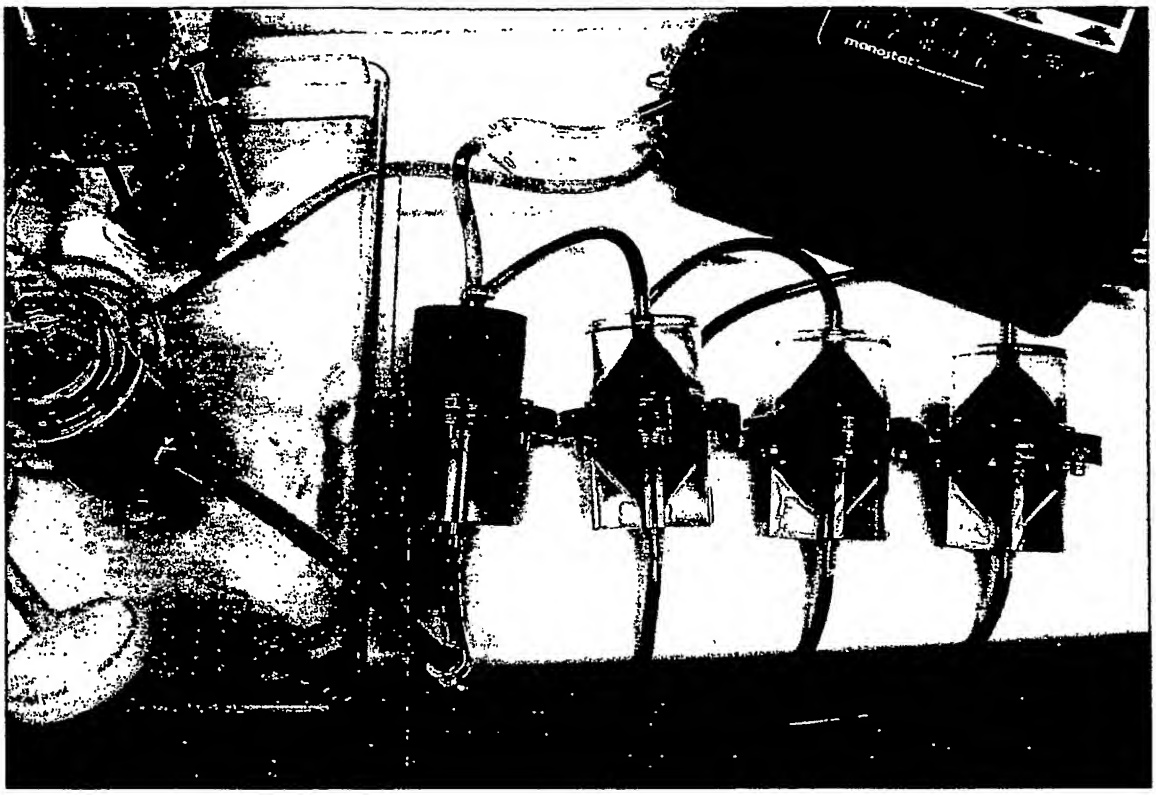

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Fig 10

Fig 6

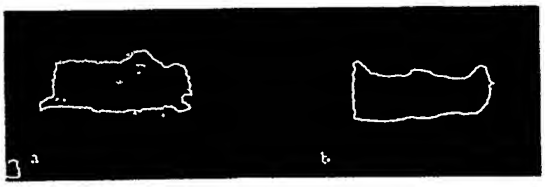

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Fig 11

Fig 12